Development of exobiopolymer-based biosensor for detection of phosphate in water

Taranpreet Kaur, Abhijit Ganguli and Moushumi Ghosh

ABSTRACT

The present study was conducted to develop a biosensor by exploiting phosphate-binding capacity of exobiopolymer (EBP) produced by *Acinetobacter* sp. An environmental isolate of EBP-producing *Acinetobacter* sp. was subjected to transposon (Tn5) mutagenesis to overproduce EBP and afford improved phosphate selectivity. A mutant producing the highest amount of EBP with high phosphate-binding capacity was selected for biosensor probe fabrication. Phosphate samples were filtered through EBP-coated membranes and phosphate retained on membranes was determined by molybdenum blue method. The color produced was read using a LED 690 nm/photodiode detection system linked to an amplifier and signals were converted to appropriate phosphate concentrations. The biosensor had a limit of detection of 0.5 mg/L and a limit of quantification 1 mg/L. The biosensor as well as the probe were found to be stable for at least 28 days. In conclusion, we believe that the biosensor may have applications in monitoring of wastewater and environmental samples. Further, the enrichment of phosphate levels by EBP can help in analysis of very low phosphate concentrations.

Key words | *Acinetobacter*, biopolymer, phosphate, sensor, transposon mutagenesis

Taranpreet Kaur
Abhijit Ganguli
Moushumi Ghosh (corresponding author)
Department of Biotechnology & Environmental Sciences,
Thapar University,
Patiala-147004,
Punjab,
India
E-mail: *mghosh@thapar.edu*

INTRODUCTION

Phosphorus (P) is ubiquitously found in living organisms and plays a very important role in their growth and maintenance. The terrestrial ecosystems derive phosphorus primarily from food while the aquatic organisms rely on dissolved phosphorus to meet their needs. In recent years it has been realized that discharge of domestic and industrial wastewater and the use of fertilizers result in increased phosphorus concentration in aquatic ecosystems (USEPA 2002). This leads to increase in algal growth resulting in eutrophication of water bodies. Therefore, it is important to develop methods for efficient removal of phosphate from water and to develop sensitive systems for monitoring phosphate levels in water.

Current remediation protocols are based on physical or chemical methods for phosphate removal and include reverse osmosis, electrodialysis, treatment with aluminum sulfate or ferric chloride, etc. (Bohdziewicz *et al.* 2003; Banu *et al.* 2008). However the physical methods result in nonspecific removal of ions while chemical methods have problems in disposal and neutralization of chemicals. Bioremediation offers an attractive alternative to physical and chemical methods due to specific intracellular accumulation of phosphate by several microorganisms as well as extracellular sequestration of phosphate by microbial biopolymers. (Cloete & Oosthuizen 2001; Liu *et al.* 2006).

Spectrophotometric assays have been the mainstay in phosphate estimation in water samples, including wastewater (APHA 2007). These assays are sensitive to small amounts of phosphorus but provide false negative results or overestimation of phosphate levels due to interference by other ions. Several modifications in conventional phosphorus estimation assays have been reported to counteract interference in phosphate estimation. However, these modifications require bulky and sophisticated instruments which are unsuitable for field measurements. The development of miniaturized sensors provides a convenient means of sample analysis at the site of collection.

Acinetobacter sp. has long been identified as a major phosphate accumulating organism in sludge and other environmental samples (Kortstee *et al.* 1994; Lin *et al.* 2003; Sathasivan 2009). Several studies have reported that *Acinetobacter* can accumulate phosphates intracellularly

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(Sathasivan 2009; Khoi & Diep 2013). Investigations in our laboratory showed that the exobiopolymer (EBP) secreted by *Acinetobacter* sp. can also sequester phosphates. The present study was conducted to exploit the phosphate-sequestering potential of *Acinetobacter* EBP for development of an analytical method for phosphate estimation. A biosensor was developed which utilizes the phosphate-binding capacity of EBP to concentrate phosphate in water samples followed by analysis with the stannous chloride method.

MATERIALS AND METHODS

Chemicals and media

Ammonium sulfate, dextrose, dipotassium hydrogen phosphate, peptone, potassium dihydrogen phosphate, yeast extract, Luria Bertani (LB) broth, kanamycin and streptomycin were purchased from HiMedia (Mumbai, India). Sodium chloride and ammonium molybdate were procured from LobaChemie (Mumbai, India). Calcium chloride and magnesium sulfate were purchased from SD-fine Chem. Ltd (Mumbai, India).

Culture

Wild and mutant *Acinetobacter* sp. were cultured in LB broth at 30 °C for routine maintenance and transferred to flocculant isolation broth medium for EBP production (Ghosh *et al*. 2009).

Mutagenesis

Antibiotic-resistant strains of donor (*Escherichia coli* containing suicidal vector pGS9) and recipient (*Acinetobacter* sp.) were selected by culturing in LB broth containing kanamycin (50 μg/mL) and streptomycin (200 μg/mL), respectively. The donor and recipient were incubated in antibiotic-containing LB broth for 18 h at 30 °C under shaking (120 rpm). For conjugal mating, donor and recipient cells were mixed in the ratio of 1:1, 1:2, 2:1, 3:1 and placed onto a filter membrane supported by luria agar, and incubated for 24 at 30 °C. The filter membrane was transferred to 50 mL LB containing double antibiotics (streptomycin 200 μg/mL and kanamycin 50 μg/mL) and incubated at 30 °C for 8 h. Each of the dilutions (0.1 mL) was spread onto luria agar selective for exconjugants.

The exconjugants were screened by examining the colony morphology and mucoid colonies were selected for further studies. The EBP overproducing mutants were selected by application of Alcian Blue assay and Sudan Black staining (Liu *et al*. 1998; Kachlany *et al*. 2001).

Extraction and purification of EBP

Extraction and purification of EBP was done by the method as described earlier (Ghosh *et al*. 2009).

Characterization of EBP

The EBP was characterized by analyzing sugars (Dubois *et al*. 1956) and amino sugars (Chaplin & Kennedy 1994).

Preparation of standard phosphate solutions

Standard phosphate solution was prepared by dissolving 100 mg of potassium hydrogen orthophosphate in 1 litre of deionized water and working concentrations (0–15 mg/L) were prepared by diluting standard solution with deionized water.

Biosensor fabrication and configuration

A miniature circuit (U1) with specific LED and sensor of 690 nm was assembled, as shown in Figure 1. The circuit was attached with a microcontroller and signals detected as milliamps. For operation, the EBP-coated membranes were subjected to colorimetry and inserted between LED and sensor. Signals received were used for subsequent processing to phosphate concentration. The assembled setup used for experiments is shown in Figure 1. The biosensor setup fits into a $20 \times 14 \times 10$ cm box and weighs about 1 kg.

Biosensor analysis of phosphate

The biosensor probe was an EBP-coated cellulose acetate membrane. The membranes (approx. 5 cm^2) were coated with 100 μL of an aqueous solution of purified EBP at EBP concentrations from 0 to 6 mg/mL. One millilitre of deionized water (for blank) or phosphate solution of concentrations ranging from 1 to 15 mg/L was passed through the coated membranes. The color of the phosphate bound with EBP coated over the membranes was developed by the addition of ammonium molybdate (250 μL) and stannous chloride (50 μL) onto the membranes (APHA 2007). The membranes were subjected to analysis by the biosensor.

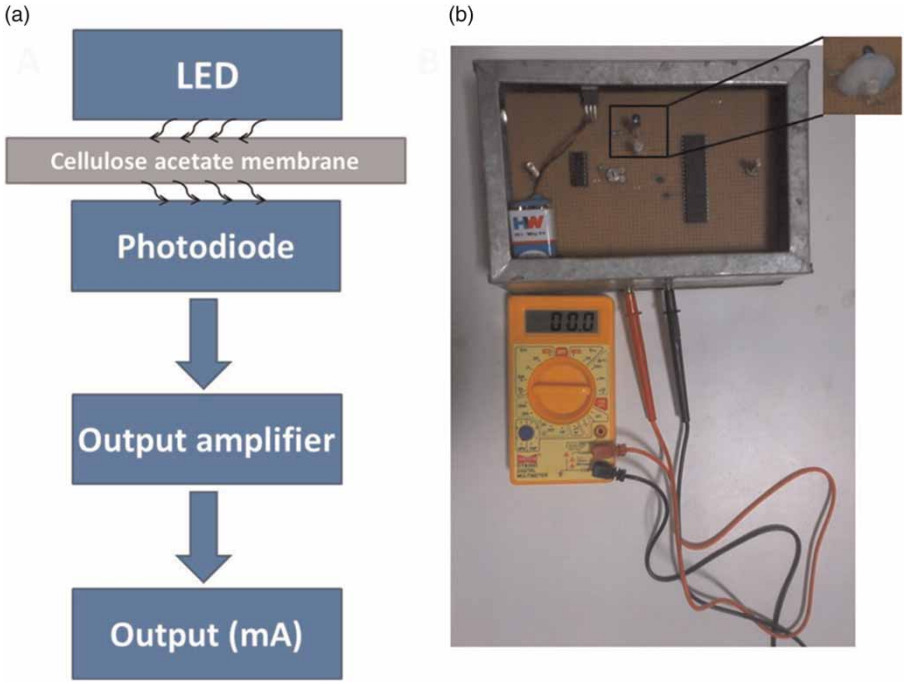

Figure 1 | (a) Schematic diagram of the phosphate biosensor. The LED emits radiations and photodiode absorbs the radiations resulting in generation of a signal. The signal intensity is proportional to color intensity on the cellulose acetate membrane. The output is amplified and the result obtained as current (mA). (b) Photograph of the phosphate biosensor setup. Inset shows cellulose acetate membrane loaded in the biosensor.

The binding efficiency of phosphate was determined by passing 1 mL of 10 mg/L phosphate solution through the membrane. The unbound phosphate remaining in the filtrate was estimated by molybdenum blue method (APHA 2007). The amount of phosphate retained on the membrane was converted to percentage of total phosphate retained on membrane and referred to as percentage phosphate binding efficiency.

Precision and accuracy

Precision or repeatability was determined by repeated analysis of spiked samples. Intra-assay variability is defined as the variability in results after repeated analysis of the same sample in one day. Standard samples in the range 1–10 mg/L were analyzed three times on the same day ($n = 5$/concentration) and percentage relative standard deviation (%RSD) was calculated.

Inter-assay variability is defined as the variability in results after repeated analysis of the sample over several days. The samples prepared for intra-day variability were analyzed for 3 consecutive days and %RSD calculated ($n = 5$/concentration). Long-term stability (4 weeks) of the sensor response was determined by analysis of samples prepared for intra-day variability on days 0, 7, 14, 21 and 28 and %RSD calculated ($n = 5$/concentration).

Accuracy or recovery refers to the closeness of the measured value to the actual value. It is calculated as (Measured concentration/Actual concentration) ×100.

Stability of biosensor probe

Cellulose acetate membranes were coated with 4 mg/mL EBP, air-dried and stored at 4 °C and ambient temperature (~25 °C) for up to 4 weeks. Membranes were used at regular intervals and phosphate concentration estimated in 10 mg/L phosphate as described above.

RESULTS AND DISCUSSION

Phosphate estimation by spectrophotometric assay is prone to interference by common environmental pollutants. Several modifications in the spectrometric methods have been developed which allow direct determination of phosphate in samples which are less susceptible to interference by environmental pollutants (Koga *et al.* 2010; Turel *et al.* 2010; Mesquita *et al.* 2011; Zimmer & Cutter 2012). Methods based

on chromatographic separation followed by spectrometric analysis of phosphates have also been reported (Rodil *et al.* 2009; Yokoyama *et al.* 2009). However, these methods use sophisticated instruments and require elaborate sample processing before analysis which makes these methods unfit for field/on-site measurements. In recent years, attempts have been made to develop phosphate biosensors using enzymes, synthetic receptors and polymers. However, a major problem associated with using enzymes is their susceptibility to poisoning/denaturation by environmental pollutants, which may lead to false negative results. Further, phosphate biosensors developed using synthetic receptors are susceptible to interference by anions commonly present in environmental samples and have not been tested for suitability for field applications (Warwick *et al.* 2013).

The present study was aimed at developing a phosphate biosensor based on the phosphate-binding capacity of EBP for two reasons. First, EBP produced by mutant strain has high selectivity for phosphate as compared to other ions. Second, phosphate binding is not substantially altered by presence of other ions. These two features ensure selectivity of phosphate retention on EBP, even in the presence of other ions/pollutants. Further, the method involves retention of phosphate during filtration through EBP-coated membrane which results in concentration of the analyte (phosphate) on membrane, thus increasing the sensitivity of the biosensor. We believe that this is the first study that reports the development of an EBP-based biosensor for phosphate estimation. Further, the method combines the advantages of selective retention of the analyte and an on-line concentration step, which has not been reported in other studies on biosensors.

Development of EBP overproducing exconjugants

The EBP production by wild type strain of *Acinetobacter* sp. is fairly low for practical applications. Hence, transposon mutagenesis was attempted to select strains producing high levels of EBP. The initial screening of mutant colonies was performed by visual inspection of colony morphology. Mucoid colonies are expected to produce EBP and, therefore selected for further screening. Based on colony morphology followed by dye staining, the mutant producing the highest amount of EBP was selected. Further, EBP from this mutant also showed the highest phosphate-binding efficiency. Hence, the mutant was selected for fabrication of the biosensor probe.

The EBP produced by mutant strain contained approximately 2-fold and 1.5-fold higher amount of sugars and amino sugars, respectively compared to EBP produced by the wild strain.

Optimization of operational conditions

The biosensor was optimized in terms of the amount of EBP coated on the membrane. Membranes were coated with 100 μL of 1–6 mg/mL of EBP and phosphate binding determined. Phosphate retention on membranes increased with an increase in the amount of EBP impregnated on membrane and reached a plateau at 4 mg/L. A further increase in EBP concentration to 5 and 6 mg/ml offered no significant increase in phosphate binding (Supplementary Figure 1, available online at http://www.iwaponline.com/wst/068/520.pdf).

The biosensor is expected to obtain a balance between sensitivity of detection/quantification and duration of analysis. All other variables remaining the same, time taken for the sample to pass through the membrane is an important factor that can impact the total time of sample analysis. Hence, the effect of EBP concentration on flow rate through the membrane was determined. The flow rate was normalized to that of uncoated membranes and expressed as percentage of flow rate through uncoated membrane. As observed in Supplementary Figure 2 (available online at http://www.iwaponline.com/wst/068/520.pdf), the flow rate through membranes remained unaffected by EBP up to a concentration of 3 mg/mL. A further increase in concentration to 4 mg/mL resulted in a slight reduction in flow rate while a further increase in EBP resulted in a sharp decline in the flow rate (Supplementary Figure 2). The observed reduction in flow rate may be attributed to blockade of membrane pores by EBP at high EBP concentrations.

Hence, considering the near-maximal binding observed at 4 mg/mL EBP and a significant reduction in flow rate at higher concentrations above 4 mg/mL, coating of membranes with 100 μL of 4 mg/mL EBP was selected for biosensor validation and sample analysis.

Calibration and validation of biosensor

One millilitre of standard phosphate solutions (1–10 mg/L) was passed through EBP-coated membranes. The membranes were treated with reagents and biosensor response was recorded as milliamperes (mA). Biosensor response was found to be linear in the range 1–10 mg/L phosphate with $R^2 > 0.99$ (Figure 2(a)). The limit of detection and limit of quantification was found to be 0.5 and 1 mg/L of phosphate, respectively.

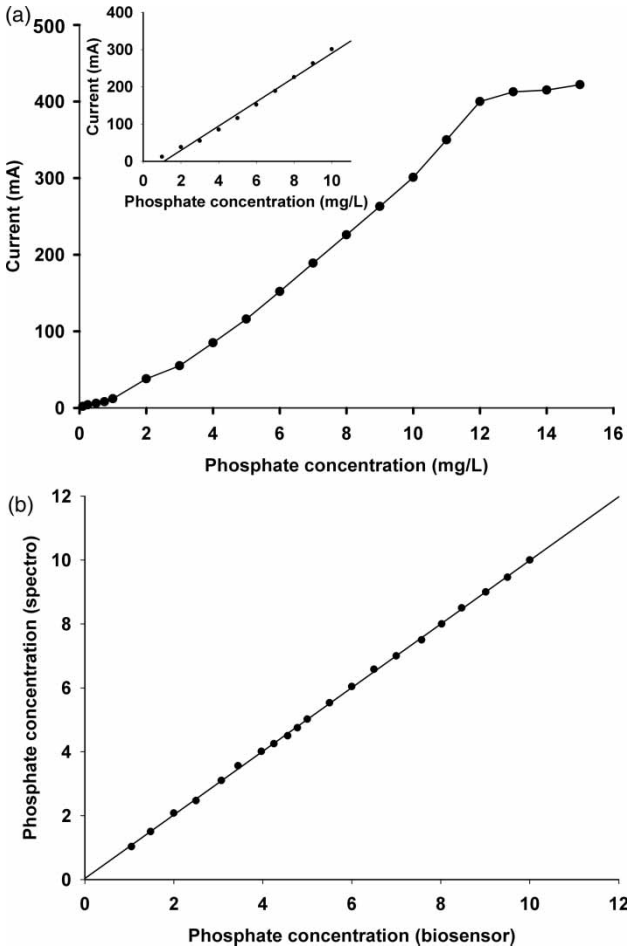

Figure 2 | (a) Calibration curve of biosensor at 0–15 mg/L phosphate concentration. Data are the mean of five samples/concentration. Inset shows regression analysis of data points in the linear range of analysis. (b) Comparison of phosphate concentration determined by biosensor and spectrophotometric method. Data are the mean of five samples/concentration.

The intra-day and inter-day variability was found to be low suggesting high precision of the biosensor (Table 1). The recovery samples were >95% at all concentrations. Further, long-term measurements over 4 weeks also showed low %RSD suggesting stability of biosensor.

Comparison of biosensor and spectrophotometric assay

The results of biosensor analysis were compared with a standard spectrophotometric method to further validate the biosensor. Standard phosphate solutions in the concentration range 1–10 mg/L were analyzed by biosensor. The same samples were also analyzed by spectrophotometric method and results obtained by both methods were compared. Regression analysis showed that phosphate concentrations determined by biosensor were in agreement with those determined by spectrophotometric method (Figure 2(b)). The coefficient of correlation was >0.99 and slope of line was close to 1 (>0.99) suggesting a good concordance between the two analytical methods.

Stability of biosensor probe

An important consideration in development of a biosensor is the long-term stability of the probe. The long-term stability of the probe is important as a large number of probes can be prepared simultaneously, offering convenience in sample analysis. Further, simultaneous preparation of probes reduces inter-analysis variations that may arise due to preparation of probes at different times.

The biosensor probes were prepared by coating membranes with 4 mg/ml EBP and stored for up to 28 days at

Table 1 | Precision, recovery and long-term stability of phosphate estimation by biosensor

	Variability (%RSD)[a]			Long-term stability[a]				
Phosphate concentration (mg/L)	Intra-day	Inter-day	% Recovery[b]	Day 0	Day 7	Day14	Day 21	Day 28
1	2.10	1.36	96.55	1.34	1.36	1.89	1.71	2.06
2	1.78	1.57	97.78	0.87	0.82	1.94	1.21	2.09
4	1.66	1.23	97.57	1.46	1.87	1.63	0.91	1.75
6	1.05	1.00	98.16	1.41	1.70	1.47	1.81	1.25
8	1.15	1.23	101.32	2.01	1.09	1.22	1.95	1.76
10	0.98	1.14	99.15	1.04	1.48	0.89	0.81	0.94

[a]Data are %RSD of five samples/concentration.
[b]Data are mean of five samples/concentration.

Table 2 | Long-term stability of biosensor probe

Storage temperature	Long-term stability[a]				
	Day 0	Day 7	Day 14	Day 21	Day 28
4 °C	1.40	1.74	2.45	1.58	2.05
Ambient	1.77	1.89	1.51	1.21	2.22

[a]Data are %RSD of five membranes/analysis.

4 °C and at ambient temperature (~25 °C). Representative membranes were drawn and 1 mL samples of 1–10 mg/L phosphate were passed. Biosensor analysis showed that the phosphate concentration determined by membranes stored for up to 28 days did not significantly differ from results obtained from freshly prepared membranes (day 0) of the same lot. The %RSD was less than 5% (Table 2).

CONCLUSION

A biopolymer-based biosensor was developed employing the molybdenum blue method for phosphate estimation. The biosensor is sensitive and accurate. Further, the biosensor is stable for long durations (at least 28 days) and does require frequent calibration. To the best of our knowledge, a biopolymer coupled to a simple electronic system with low power consumption and reproducibility has not yet been reported for detecting phosphate. The superiority of the biopolymer lies in concentrating phosphate in its matrix on account of its high affinity for phosphate and the bound phosphate is amenable to colorimetry, rendering the EBP sensor suitable in areas with low phosphate concentration. The applicability of biosensor can be extended for monitoring of environmental, industrial and household wastewater analysis.

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REFERENCES

APHA-AWWA-WPCF 2007 *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, DC, USA.

Banu, R. J., Do, K. U. & Yeom, I. T. 2008 Phosphorus removal in low alkalinity secondary effluent using alum. *International Journal of Environmental Science and Technology* **5** (1), 93–98.

Bohdziewicz, J. E. & Sroka, I. 2003 Application of ultrafiltration and reverse osmosis to the treatment of the wastewater produced by the meat industry. *Polish Journal of Environmental Studies* **12** (3), 269–274.

Chaplin, M. F. & Kennedy, J. F. 1994 *Carbohydrate Analysis: A Practical Approach*. ILR Press, Oxford, New York.

Cloete, T. E. & Oosthuizen, D. J. 2001 The role of extracellular exopolymers in the removal of phosphorus from activated sludge. *Water Research* **35** (15), 3595–3598.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. 1956 Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 350–356.

Ghosh, M., Ganguli, A. & Pathak, S. 2009 Application of a novel biopolymer for removal of *Salmonella* from poultry wastewater. *Environmental Technology* **30** (4), 337–344.

Kachlany, S. C., Levery, S. B., Kim, J. S., Reuhs, B. L., Lion, L. W. & Ghiorse, W. C. 2001 Structure and carbohydrate analysis of the exopolysaccharide capsule of *Pseudomonas putida* G7. *Environmental Microbiology* **3** (12), 774–784.

Khoi, L. Q. & Diep, C. N. 2013 Isolation and phylogenetic analysis of polyphosphate accumulating organisms in water and sludge of intensive catfish ponds in the Mekong Delta, Vietnam. *American Journal of Life Sciences* **1** (2), 61–71.

Koga, M., Matsuoka, S. & Yoshimura, K. 2010 Improved solid-phase spectrometry for the microdetermination of total and dissolved phosphate. *Analytical Sciences* **26** (9), 963–968.

Kortstee, G. J., Appeldoorn, K. J., Bonting, C. F., van Niel, E. W. & van Veen, H. W. 1994 Biology of polyphosphate-accumulating bacteria involved in enhanced biological phosphorus removal. *FEMS Microbiology Review* **15** (2–3), 137–153.

Lin, C. K., Katayama, Y., Hosomi, M., Murakami, A. & Okada, M. 2003 The characteristics of the bacterial community structure and population dynamics for phosphorus removal in SBR activated sludge processes. *Water Research* **37** (12), 2944–2952.

Liu, M., Gonzalez, J. E., Willis, L. B. & Walker, G. C. 1998 A novel screening method for isolating exopolysaccharide-deficient mutants. *Applied and Environmental Microbiology* **64** (11), 4600–4602.

Liu, Y. N., Xue, G., Yu, S. L. & Zhao, F. B. 2006 Role of extracellular exopolymers on biological phosphorus removal. *Journal of Environmental Sciences (China)* **18** (4), 670–674.

Mesquita, R. B., Ferreira, M. T., Toth, I. V., Bordalo, A. A., McKelvie, I. D. & Rangel, A. O. 2011 Development of a flow method for the determination of phosphate in estuarine and freshwaters–comparison of flow cells in spectrophotometric sequential injection analysis. *Analytica Chimica Acta* **701** (1), 15–22.

Rodil, R., Quintana, J. B., Lopez-Mahia, P., Muniategui-Lorenzo, S. & Prada-Rodriguez, D. 2009 Multi-residue analytical method for the determination of emerging pollutants in water by solid-phase extraction and liquid chromatography-tandem

mass spectrometry. *Journal of Chromatography A* **1216** (14), 2958–2969.

Sathasivan, A. 2009 Biological phosphorus removal processes for wastewater treatment. In: *Encyclopedia of Life Support Systems, Water and Wastewater Treatment Technologies* (S. Vigneswaran, ed.). Encyclopedia of Life Support Systems (EOLSS) Publishers, Oxford, UK, pp. 1–23.

Turel, M., Duerkop, A., Yegorova, A., Karasyov, A., Scripinets, Y. & Lobnik, A. 2010 Microtiter plate phosphate assay based on luminescence quenching of a terbium complex amenable to decay time detection. *Analytica Chimica Acta* **675** (1), 42–48.

USEPA 2002 Epa Ground Water & Drinking Water – Secondary Drinking Water Regulations: Guidance for Nuisance Chemicals, http://water.epa.gov/drink/contaminants/secondarystandards.cfm (accessed 21 July 2013).

Warwick, C., Guerreiro, A. & Soares, A. 2013 Sensing and analysis of soluble phosphates in environmental samples: a review. *Biosensors and Bioelectronics* **41**, 1–11.

Yokoyama, Y., Danno, T., Haginoya, M., Yaso, Y. & Sato, H. 2009 Simultaneous determination of silicate and phosphate in environmental waters using pre-column derivatization ion-pair liquid chromatography. *Talanta*. **79** (2), 308–313.

Zimmer, L. A. & Cutter, G. A. 2012 High resolution determination of nanomolar concentrations of dissolved reactive phosphate in ocean surface waters using long path liquid waveguide capillary cells (LWCC) and spectrometric. *Limnology and Oceanography: Methods* **10**, 568–580.

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